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Human Tumor Necrosis Factor-α Receptor

PURIFICATION BY IMMUNOAFFINITY CHROMATOGRAPHY AND INITIAL CHARACTERIZATION*

Exhibit E

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The receptor for human tumor necrosis factor-α (TNF-α) was isolated from a subclone of the human histiocytic lymphoma cell line U937. These cells exhibit a single class of high affinity receptors (K_d = 0.51 ± 0.25 nM) with an average density of 55,000 ± 5,000 binding sites/cell. After solubilization with detergent, the receptor retained its ability to bind free TNF-α but failed to bind to TNF-α immobilized on various solid supports. For receptor purification, ^{125}I-TNF-α was covalently attached to the receptor on intact cells by the bifunctional cross-linking reagents ethylene glycolbis(succinimidylsuccinate) or 3,3-dithiobis(sulfosuccinimidylpropionate). The cells were then solubilized with the nonionic detergent Triton X-100, and the supernatants, clarified by centrifugation, were passed over an IgG-Sepharose column prepared from TNF-α antiserum. The receptor-rich fraction from the antibody column was further purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These two steps together provided approximately 165,000-fold purification of the TNF-α receptor. The TNF-α receptor-ligand complex obtained by this method had a subunit molecular weight of 100,000 ± 5,000 when examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis but on gel filtration the complex migrated with an apparent molecular weight of 480,000 ± 32,000. However, the receptor showed a molecular weight of 65,000 ± 32,000 when gel filtration was performed in the absence of ligand. Additional characteristics of the receptor are discussed.

Human tumor necrosis factor α (TNF-α)[1] is a nonglycosylated protein with a molecular weight of 17,350 which was originally purified from culture supernatants of the promyelocytic leukemia cell line HL60 induced by 4β-phorbol 12-myristate 13-acetate (1). This cytokine is now available as a recombinant DNA product from *Escherichia coli* (2). Although initially identified by its ability to induce hemorrhagic necrosis of transplantable tumors *in vivo* (3), TNF-α has been found to have a variety of actions *in vitro*, including the inhibition of proliferation of tumor cells, the stimulation of proliferation of fibroblasts (4), enhancement of phagocytosis and cytotoxicity in polymorphonuclear neutrophils (5, 6), and increased adherence of neutrophils to cultured endothelial cells (7). It has been shown to promote procoagulant activity (8) and the expression of class I major histocompatibility complex antigens on endothelial cells (9). TNF-α inhibits the synthesis of lipoprotein lipase in cultured adipocytes such as 3T3-L1 (10, 11), whereas it has been shown to increase the levels of this enzyme in liver cells (12). These and other effects of TNF-α have been the subject of several recent reviews (13–15).

The mechanism by which TNF-α affects such a wide variety of cells is not understood. A single class of high affinity receptors with a K_d of 0.1–1.0 nM has been identified on a variety of cell lines (4, 6, 16–21). With the exception of red blood cells (6), most cells thus far examined have been shown to possess some level of TNF-α receptors. The number of receptors among different cell types varies widely from as low as 200/cell to as many as 50,000/cell. The receptor numbers can be upregulated by interferon-γ (18, 20, 22) and Concanavalin A (23), and down-regulated by interleukin-1 (24), metabolic inhibitors (20), and phorbol esters (24–26). Clearly, the binding of TNF-α to cell surface receptors is the first step in the mechanism of its action. However, the time course of biological activity (18–20 h at 37 °C) (27) is much slower than the time course of binding (30 min at 37 °C) (21). It appears that the presence of the TNF-α receptor alone, although necessary, is not sufficient for generating various cellular responses.

In an effort to learn about the mechanism of TNF-α action, we have isolated and partially characterized the TNF-α receptor from a subclone of the human histocytic cell line U937. The receptor can be extracted from the cells by treatment with various detergents while maintaining full binding activity. Furthermore, the receptor can be purified to near homogeneity by a combination of immunoaffinity chromatography and preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

EXPERIMENTAL PROCEDURES[2]

RESULTS

Binding Characteristics of the TNF-α Receptor—The binding of ^{125}I-TNF-α to intact U937 cells was examined using

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[1] The abbreviations used are: TNF-α, tumor necrosis factor-α; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IMDM, Iscove's modified Dulbecco's medium; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate); EGS, ethylene glycolbis(succinimidylsuccinate); sulfo-EGS, ethylene glycolbis(sulfosuccinimidylsuccinate); Thesit, [dodecylpoly(ethylene glycol ether)$_n$]; DSS, disuccinimidyl suberate; DSP, dithiobis(succinimidylpropionate); DTSSP, 3,3-dithiobis(sulfosuccinimidylpropionate); SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; PBS, phosphate-buffered saline; GuHCl, guanidine hydrochloride; PMSF, phenylmethylsulfonyl fluoride; FBS, fetal bovine serum; PEG 6000, polyethylene glycol 6000; hGH, human growth hormone; FPLC, fast protein liquid chromatography; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin.

[2] The "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

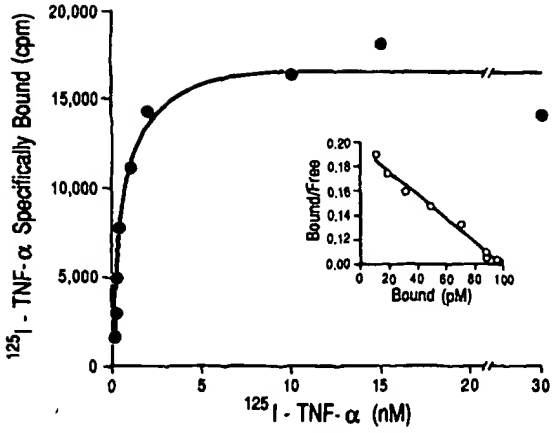

FIG. 1. **Specific binding of ^{125}I-TNF-α to U937 cells.** 10^5 cells were incubated at 4 °C for 2 h with increasing amounts of labeled ligand in the presence or absence of a 100-fold excess unlabeled ligand. Thereafter, the cells were washed three times and counted for cell-bound radioactivity. Each point is an average of triplicate determination. The Scatchard analysis of the data is shown in the *inset*.

TABLE I

Solubilization of TNF-α receptor with various detergents

Approximately 10^7 cells were suspended in 0.1 ml of 50 mM Tris, pH 7.5, containing 1% detergent and aprotinin (0.2 mg/ml), 1 mM phenylmethylsulfonyl fluoride, and 0.1% bacitracin. After diluting the extracts 10-fold with the same buffer without detergent, the solubilized receptor was collected as the supernatant after centrifugation. By using the soluble binding assay, the amount of receptor solubilized by each detergent was estimated, assuming a ligand receptor binding stoichiometry of 1:1. The results are expressed as picomoles of TNF-α bound. All determinations were made in triplicate.

Detergent	^{125}I-TNF-α
	pmol/10^7 cells
Triton X-100	3.1 ± 0.3
Sodium deoxycholate	3.4 ± 0.8
CHAPS	1.9 ± 0.3
n-Octyl glucopyranoside	3.3 ± 0.6
Digitonin	3.7 ± 0.5
Thesit	2.1 ± 0.4

various concentrations of labeled ligand either in the presence or absence of excess unlabeled ligand (Fig. 1). The specific binding of TNF-α increased with increasing amount of labeled ligand added and reached saturation at 10 nM. The Scatchard plot of the binding data (*inset*, Fig. 1) was consistent with a single class of high affinity binding sites having a K_d of approximately 0.5 nM and approximately 59,000 binding sites/cell.

The TNF-α receptor could be solubilized by a variety of detergents with apparent full retention of binding activity. As shown in Table I, the recovery of soluble binding activity was similar for most of the detergent extracts tested, (~3 pmol/10^7 cells), yielding approximately three times the number of sites detected on the cell surface, although somewhat lower activities were observed using the detergents CHAPS or Thesit. All subsequent solubilizations of cells were done using 1% Triton X-100. Using this detergent, a curvilinear Scatchard plot was obtained for the solubilized receptor (Fig. 2, insert). A high affinity component corresponding to a K_d of approximately 0.4 nM was observed, and these sites were present in approximately one-half the number (27,000 sites/cell) observed on the cell surface. The remaining sites solubilized were present at roughly 10-fold the concentration seen on the intact cell surface, but the affinity of these sites was much lower (~15 nM) than for nonsolubilized receptor.

Strategy for TNF-α Receptor Purification—The receptor,

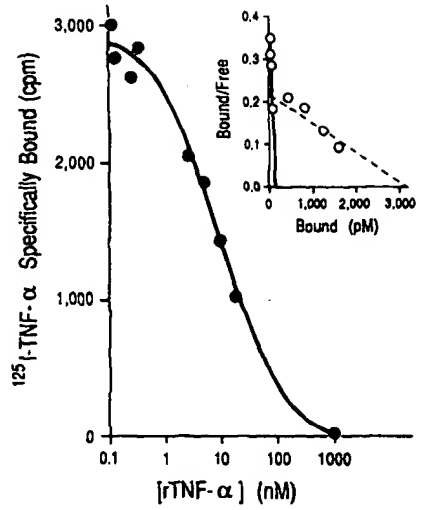

FIG. 2. **Competition curve of ^{125}I-TNF-α with unlabeled TNF-α for solubilized receptor isolated from U937 cells.** U937 cells were solubilized with 1% Triton X-100 and protease inhibitors as described under "Experimental Procedures" (Miniprint). The extract was diluted 1/10 with phosphate-buffered saline and then assayed with 0.1 nM ^{125}I-TNF-α in the absence or presence of unlabeled ligand at various concentrations. After 30 min at 37 °C, the receptor-ligand complex was precipitated with bovine γ-globulin and polyethylene glycol and then specifically bound TNF-α was calculated as outlined under "Experimental Procedures." The *inset* shows a Scatchard plot of this data.

solubilized as described above, bound specifically to free TNF-α but failed to show binding to TNF-α immobilized by amino or carboxyl linkages on agarose or other supports.[3] However, these same affinity resins were able to bind anti-TNF-α antibodies. This difference may be because the coupling procedure modifies amino or carboxyl groups on the ligand which are essential for receptor binding, but less important for binding to the antibodies. Loss of ligand binding was also observed using the heterobifunctional cross-linker *N*-succinimidyl-3-(2-pyridyldithio)propionate under conditions where only lysines or the amino terminus would be modified. The TNF-α binding activity in crude cell extracts bound to various lectin affinity resins including Concanavalin A-Sepharose, lentil lectin-Sepharose, and wheat germ agglutinin-Sepharose. However, this activity was recovered in low yield (5–10%) and with a specific activity comparable to that of the starting material (data not shown). Since these direct approaches toward receptor purification proved ineffective, an alternate strategy was adopted whereby TNF-α was covalently attached to its receptor, and the receptor-ligand complex was purified by anti-TNF-α immunoaffinity chromatography and preparative SDS-PAGE.

Cross-linking of the Receptor to TNF-α—TNF-α bound to cell surface receptors was covalently attached using various bifunctional cross-linking reagents including EGS, sulfo-EGS, disuccinimidyl suberate, DSP, and DTSSP. The cross-linked receptor-ligand complex was solubilized by Triton X-100 and analyzed by SDS-PAGE (Fig. 3). An autoradiogram of this gel is shown in the *upper panel* of Fig. 3. A band at approximately 95 kDa was observed only when the cells were incubated with labeled TNF-α alone, but absent when an excess of unlabeled TNF-α was also included. When incubated with a ligand concentration at the K_d (0.5 nM), approximately 50% of the sites were cross-linked at 100 kDa. This was in

[3] These matrices include Affi-Gel 10, 15, 102, 202, and FMP-activated Avid gel T, and Beckman Ultra EP and Reacti-Gel (6X), (HW-65F), and (25DF) (Pierce).

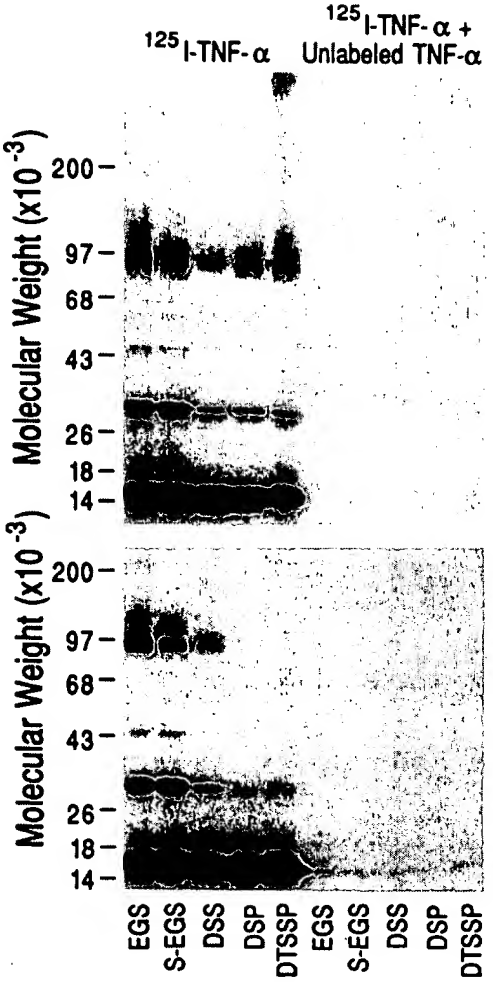

FIG. 3. **Analysis of the receptor-ligand complex after treatment with various bifunctional cross-linking reagents.** In each case, 10^7 cells, bound to ^{125}I-TNF-α, were treated at 4 °C with freshly prepared cross-linking reagents (0.1 mM final) for 40 min. Afterwards, the reaction was quenched and the cells solubilized as described under "Experimental Procedures." Samples were resolved by SDS-PAGE (5–15% acrylamide gradient) under nonreducing (*upper panel*) or reducing conditions using a final concentration of 50 mM dithiothreitol (*lower panel*). The receptor-ligand complex was then visualized by autoradiography. *DSS*, disuccinimidyl suberate.

agreement with the amount of ligand bound to cells at this concentration. The two other major bands at 17,000 and 34,000 and a minor band at approximately 45,000 daltons represent monomeric, dimeric, and trimeric forms of TNF-α since they appeared regardless of the cross-linking reagent used, and even in the absence of receptor. The 95-kDa band was most intense when EGS or DTSSP was used. The efficiency of cross-linking as determined in several experiments by the amount of ^{125}I-labeled TNF-α appearing at 95–100 kDa was 5–10% in the case of EGS and DTSSP. The other reagents showed lower efficiencies and were not investigated further. When the gel was run under reducing conditions, the TNF-α-receptor complex linked by the disulfide containing reagents dithiobis(succinimidylpropionate) and DTSSP was dissociated (*lower panel*, Fig. 3). The three noncleaved complexes seen in the first three lanes migrate with slightly higher molecular masses (approximately 100 kDa) upon reduction, most likely the result of breaking the intramolecular disulfide bonds. Due to the reversible nature of DTSSP under reducing conditions, this cross-linker was used to identify purified receptor, free of ligand, as described below.

Immunoprecipitation of the TNF-α Receptor Complex—The cross-linked ligand-receptor complex bound to rabbit anti-TNF-α antibodies and could be immunoprecipitated with protein A-Sepharose and recovered by extraction with SDS sample buffer as shown in Fig. 4. In this experiment, the cross-linking reagent was EGS. The *left panel* shows the Coomassie-stained gel of two immunoprecipitates. In the first case, the TNF-α receptor complex was incubated with anti-TNF-α antibodies (*lane 1*). In the second case, the antibody was against human growth hormone (*lane 2*). The protein patterns for both samples appear identical. However, as shown by the autoradiogram of this gel (*right panel*), only anti-TNF-α was capable of precipitating the receptor-ligand complex (as well as free ligand) (*lane 1*). The specificity of this procedure is demonstrated by the absence of any radiolabeled proteins in *lane 2*.

Immunoaffinity Chromatography—Based on the results of the immunoprecipitation, a method was devised to purify cross-linked receptor on a larger scale. Polyclonal anti-TNF-α antibodies, which were shown to immunoprecipitate the cross-linked receptor complex, were coupled to CNBr-activated Sepharose (~1 mg/ml). Approximately 40% of the label but less than 5% of the protein in a given cross-linked cell extract bound to the anti-TNF-α-Sepharose. Although the percentage of bound label could be increased by increasing the ratio of affinity matrix to extract, we observed that the material recovered from these columns was of lower purity than when the label was present in excess (data not shown). As shown in Table II, 80% of the receptor which had bound to the affinity column could be recovered by elution with 6 M guanidine HCl giving an overall yield of 32% for the affinity column step. (For the experiment shown, the amount of covalently attached TNF-α was 1.7%.) This provided a 210-fold purification. Fig. 5 shows the SDS-PAGE analysis of a typical affinity purification. The crude cell extract (Fig. 5*A*,

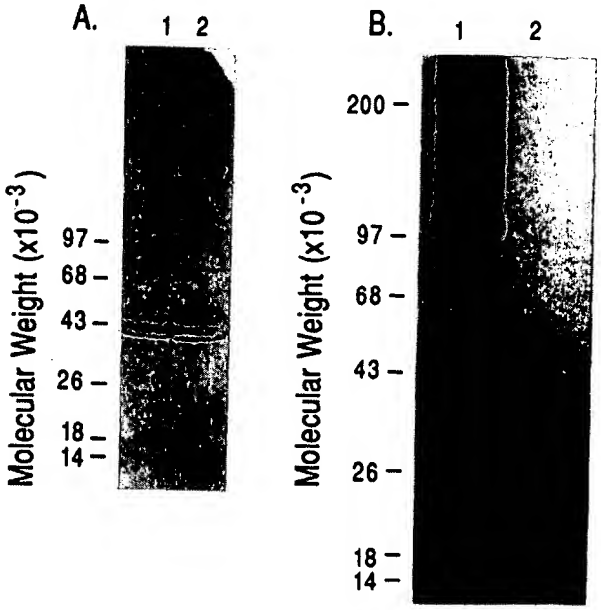

FIG. 4. **Immunoprecipitation of cross-linked TNF-α-receptor.** The solubilized receptor-ligand complex was first incubated with either anti-TNF-α or anti-human growth hormone antisera as described under "Experimental Procedures." The immune complexes were precipitated with protein A bound to Sepharose beads. The resulting pellets were extracted with SDS sample buffer and resolved on a 5–15% acrylamide gradient SDS-PAGE. The Coomassie-stained gel is shown in *panel A*. *Lane 1* corresponds to the immunoprecipitate using anti TNF-α antibodies, whereas *lane 2* shows the material precipitated with anti-human growth hormone. The autoradiogram of these bands is shown in *panel B*.

TABLE II
Purification of TNF-α receptor

Fraction	Protein	TNF-α[a]	Specific activity	Purification	Recovery
	mg	*pmol cross-linked*	*fmol/mg*	*-fold*	*%*
Cross-linked[b] cell extract	313[c]	3.8	12.1		100
Antibody column eluate	0.47[c]	1.7	2,553	211	32
SDS-PAGE gel slice	0.0002[d]	0.4	2,000,000	165,290	11

[a] This value was calculated based on the measured amount of ^{125}I-TNF-α in the extract, and the observed percentage (1.7) of this label which runs at 100,000 daltons by SDS-PAGE.
[b] 1.5×10^{10} cells were first homogenized at 4 °C using a Tekmar homogenizer at 25% maximum speed for 5 × 20-s bursts. The crude membranes were collected by centrifugation at 200,000 × *g* for 15 min. These membranes were solubilized as described under "Experimental Procedures" (Miniprint) for whole cells.
[c] Protein concentration was determined by a modification of the method of Bradford (31).
[d] Protein was estimated by comparing the silver stain intensity of the 100-kDa band run on a second gel (not shown) with known amounts of standards. The intensity was measured using an LKB 2202 Ultrascan laser densitometer.

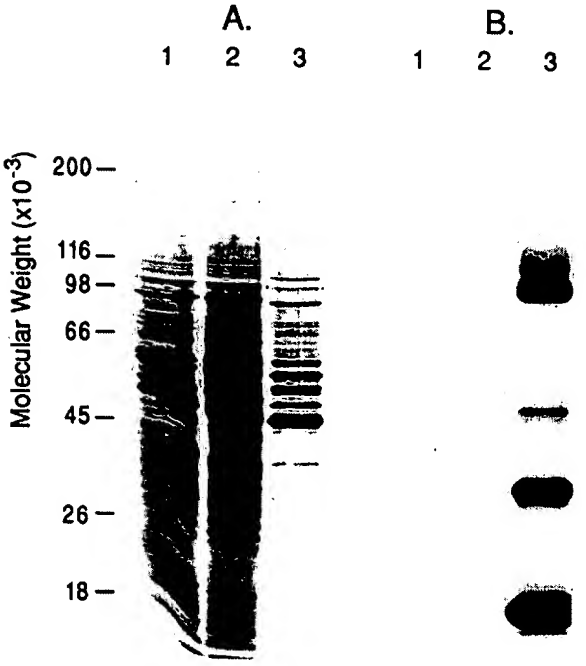

FIG. 5. **SDS-PAGE analysis of immunoaffinity purified cross-linked ^{125}I-TNF-receptor.** Various fractions from the immunoaffinity purification were subjected to SDS-PAGE on a 5–15% acrylamide gradient. An equal volume (5 μl) of the column load (*lane 1*) and the unabsorbed fraction (*lane 2*) were applied to the gel. Following dialysis and acetone precipitation, the column eluate was resuspended in a total volume of 70 μl. Approximately 10% of this sample was applied to the gel (*lane 3*). *Panel A* is the silver-stained gel. The autoradiogram of this gel is shown in *panel B*.

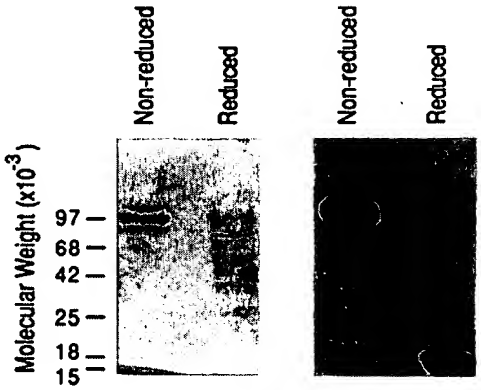

FIG. 6. **SDS-PAGE autoradiography of cross-linked receptor recovered from a polyacrylamide gel.** Cross-linked (DTSSP) receptor which had been partially purified by the anti-TNF affinity column was further purified by SDS-PAGE (5–15% acrylamide gel). The 100-kDa band was sliced from the frozen gel, eluted by diffusion, and analyzed by a second SDS-PAGE under nonreduced (*lane 1*) or reduced (*lane 2*) conditions. *Left panel* is the silver stained gel, and *right panel* is the autoradiogram of that gel.

lane 1) has a very similar silver staining pattern to that of the nonbinding fraction (*lane 2*). The eluate from the affinity column shown in *lane 3* indicates removal of several contaminating proteins. Most of the contaminants remaining in the affinity column eluate migrate between 45 and 66 kDa. The region of the gel corresponding to the receptor-ligand complex is identified by the autoradiogram of this gel (Fig. 5*B*, *lane 3*). In addition to the band appearing at 100 kDa, a more diffuse band appears just above it. This is also seen in concentrated samples of the starting material (Fig. 4*B*) and may represent heterogeneity of the receptor-ligand complex. This panel also demonstrates that the affinity column serves as a concentration step for the labeled proteins.

Preparative SDS-PAGE—Eluates from the affinity column were further purified by SDS-PAGE. After electrophoresis, the gels were immediately frozen, sliced, and eluted. Ninety-five percent of the labeled protein migrating at a M_r of 100,000 was recovered from the slice by extraction with 0.01% SDS at 4 °C for 12 h. As shown in Table II, this step provides an additional purification of approximately 780-fold and an overall purification of 165,000-fold. Although the yield from the gel slice was very often close to 100%, the preceding steps (concentration of the affinity column eluate and application to the preparative gel) were less efficient and account for the overall recovery of 11%. Additionally, when cells were cross-linked on a preparative scale, less receptor (~50%) was covalently coupled, in part because of the increased time required in between steps to wash and spin down large amounts of cells. Finally, detergent solubilization of the labeled receptor had a typical yield of around 50%. These factors combined account for the yield of less than 1 pmol of cross-linked receptor from 1.5×10^{10} cells.

Fig. 6 shows the SDS-PAGE analysis of purified complex isolated as described above. The TNF-α was attached to the receptor using the reversible cross-linker DTSSP. The *left panel* shows the silver staining pattern of the gel. Under nonreducing conditions, the complex migrates with a molecular mass of approximately 100 kDa. However, when the sample was run under reducing conditions, the majority of silver-stained protein appeared at 85 kDa. Some additional

material remains in the 100,000-dalton region and most likely represents a contaminant which has copurified with the receptor-ligand complex. There are also some minor bands staining between the 42- and 97-kDa markers. These most likely correspond to proteolytic fragments of the receptor and/or contaminants which are released when disulfide bonds are broken. As seen in the autoradiogram to the right of the gel, the reduction in size under reducing conditions coincides with the cleavage of TNF-α from its receptor. The radioactively labeled band migrated to the position of free TNF-α (17,000 dalton), as expected. At this concentration (~1 ng), TNF-α itself was not stained by silver. These data suggest that the TNF-α binding subunit has a molecular weight of approximately 85,000 and that the ligand binds to this subunit in a 1:1 stoichiometry.

Gel Filtration Analysis of TNF-α Receptor—The molecular weights of the solubilized receptor-ligand complex and the solubilized free receptor were also determined by gel filtration using a Superose 6 column on a fast protein liquid chromatography system (Fig. 7). The ligand-receptor complex showed three major peaks of radioactivity at M_r 20,000, 230,000, and 480,000 (Fig. 7*A*). When the peaks of the elution were subjected to analysis by SDS-PAGE, only the peak at 480,000 (*peak a*), contained the 100,000 dalton labeled protein (*inset*, Fig. 7, *lane a*). The two remaining peaks, (*peaks b* and *c*), contained dimeric and monomeric TNF-α. The position of *peak b* seems to indicate that monomers and dimers of TNF-α are noncovalently associated with an additional protein, possibly receptor. The profile of unbound receptor is illustrated in the *lower portion* of Fig. 7. Although the binding activity of the solubilized receptor was distributed over a broad range of molecular mass, the peak was centered around 65,000 daltons. A shoulder to the left of the peak suggests the existence of a higher molecular weight species of the receptor. Since the amount of cell extract applied to the column was equal to that used to analyze the cross-linked preparations, the increased broadness of this peak is interpreted to represent a reversible association with additional subunits/proteins, rather than simply an overloading of the column.

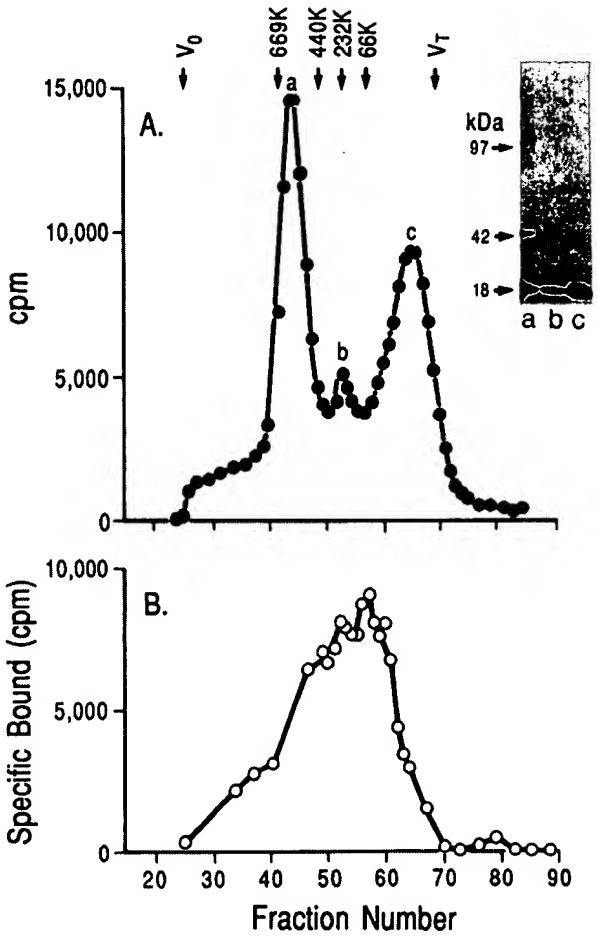

FIG. 7. **Gel filtration of the TNF-α receptor by Superose 6 fast protein liquid chromatography.** Approximately 10^8 U937 cells were prebound with ligand and then cross-linked with DTSSP. They were then solubilized (10^8 cells/ml) with 1% Triton X-100 in the presence of protease inhibitors as described under "Experimental Procedures." Two hundred microliters of extract were applied to a Pharmacia LKB Biotechnology Inc. Superose 6 column equilibrated with 0.1% Triton X-100 in phosphate-buffered saline at room temperature. The flow rate was 0.3 ml/min/fraction. Each fraction was then counted directly in a γ-counter (●). Each of the three peak fractions (*peaks a, b,* and *c*) was subsequently analyzed by SDS-PAGE and autoradiography (*inset*). Another 10^8 cells were solubilized directly without prior exposure to ligand and resolved by gel filtration. Each fraction was then assayed for TNF-α binding as described under "Experimental Procedures." The column was calibrated using globular proteins of known molecular weight: thyroglobulin (669,000), ferritin (440,000), catalase (232,000), and bovine serum albumin (66,000).

DISCUSSION

In the present study, we report the partial purification of the receptor for TNF-α. This was accomplished by covalently attaching the ligand to the receptor on intact cells, solubilizing the complex with detergent, and then isolating it by means of an anti-TNF-α immunoaffinity column. A subclone of the human histocytic cell line U937 was chosen as the source for the receptor because it expresses a relatively large number of single class, high affinity receptors, and these cells are convenient to grow in large quantities. Various detergents were employed to solubilize the cells in an attempt to recover maximal binding activity. It is difficult to compare the yields, since in all cases a greater amount of TNF-α binding was observed in the detergent extract compared to the intact cells. Much of the additional binding seems to be of lower affinity than that observed on the cell surface. This increased binding could be due to exposure of cryptic binding sites upon detergent solubilization. It has been shown previously that the density of TNF-α receptors on the surface of the cell can be increased or decreased by treatment with lectins or phorbol esters respectively (23, 25). When cells were treated with Concanavalin A in the presence of the *de novo* protein synthesis inhibitor cyclohexamide, there was a gradual increase in the number of binding sites on the surface, suggesting a cytoplasmic pool of receptors. Likewise, when surface receptors were depleted by treating the cells with phorbol esters, such as 4β-phorbol 12-myristate 13-acetate, ligand binding could be recovered by solubilizing the cells with detergent in approximately equivalent amounts as solubilized from untreated cells. These internal receptors probably account for the cryptic sites.

Several attempts were made to further purify the solubilized TNF-α receptor using affinity columns made by immobilizing the ligand to a variety of supports. None of the resins were able to bind significant quantities of receptor. Therefore, an approach was taken which relied on immunoaffinity purification, followed by preparative SDS-PAGE.

The technique of immunoaffinity chromatography using an antibody directed against the ligand has been used successfully for the isolation of several membrane receptors, including the receptors for parathyroid hormone (32), lutropin/choriogonadotropin (33), and interferon-β receptor (34). One

of the major limitations of this technique is that the yield is directly proportional to the amount of ligand which is covalently attached to the receptor. Therefore, one must find a reagent which provides the highest cross-linking efficiency of the receptor to the ligand. Several cross-linking reagents were examined for their ability to attach ^{125}I-TNF-α to its receptor. In each case, a labeled complex was formed having an approximate molecular weight of 95,000–100,000 by SDS-PAGE (Fig. 3). EGS and DTSSP had the highest efficiency for cross-linking (10%) TNF-α to the receptor on the surface of U937 cells. On occasion, a variable amount of a labeled band with a molecular weight of about 75,000 was also observed (data not shown), as has been reported by others (35, 36). However, the relative abundance of this band was diminished by conditions which minimized proteolysis. When cross-linked receptor was stored at room temperature for several hours, or at 4 °C for extended periods, an increasing amount of the M_r 75,000 peptide could be detected which was correlated with a decreasing amount of the M_r 100,000 peptide (data not shown). The cross-linked TNF-α receptor could be further purified by using anti-TNF-α immunoaffinity chromatography.

Approximately 40–50% of the cross-linked and solubilized TNF-α receptor complex bound to the immunoaffinity column. It is not clear under these conditions what fraction of noncross-linked TNF-α receptor complex also bound to the column, although a portion of this material may be lost during the extensive washing that precedes elution. The recovery of labeled protein from the column was typically 70–80% of that which bound. However, as seen in Fig. 5, the ligand-receptor complex obtained at this stage was only partially purified. The silver staining pattern illustrates that most of the protein in the eluate migrates below 55,000 daltons by SDS-PAGE, and this enabled us to use SDS-PAGE as a second purification step. The combined purification by both immunoaffinity chromatography and preparative SDS-PAGE was approximately 165,000-fold. The overall yield for these two steps was estimated to be about 34 ng of receptor from approximately 1.5×10^{10} U937 cells based on the content of ^{125}I-labeled TNF-α in the product. The cross-linked receptor is approximately 20% pure at this point (This calculation assumes a comparable binding and cross-linking efficiency for both labeled and unlabeled TNF-α.)

The molecular weight of the solubilized receptor was also determined by gel filtration chromatography. We compared the chromatographic mobility of the cross-linked receptor-ligand complex to that of the free receptor. When the former species was analyzed by gel filtration, the labeled complex migrated with an apparent molecular weight of 480,000. When examined by SDS-PAGE, the M_r 480,000 region of the gel filtration eluate does not produce any labeled bands larger than 100,000 daltons. This suggests that the larger molecular weight species seen with gel filtration is the result of a reversible association rather than cross-linking itself, since the bonds formed by DTSSP should not be cleaved by SDS. Part of this increase may be due to association with detergent micelles. The native molecular weight reported here is somewhat larger than the M_r of 360,000 which had been reported previously using murine L(S) and human HeLa S_2 cells, using the same detergent (35). There are several possible explanations for this discrepancy. This difference may be due to species differences in the TNF-α ligand and the cell lines, as well as a different cross-linking reagent used by the previous investigators. In addition, a different gel (AcA 34 *versus* Superose 6) and a lower column temperature (4 °C) were used in that study. In the study using L(S) and HeLa S_2 cells (35), it was indicated that labeled protein isolated from the high molecular weight fractions of the gel filtration column did not enter the SDS gel. We did not observe this effect and this difference may reflect a more selective cross-linking limited to TNF-α and its binding site.

When the unbound TNF-α receptor was solubilized from the cell with Triton X-100 and examined by gel filtration, the peak of binding activity comigrated with the bovine serum albumin standard (M_r 66,000). This size is similar to that estimated by others using cross-linking studies (16, 17, 35, 36). A small amount of TNF-α binding corresponding to a higher molecular weight was also observed as a shoulder on the elution profile (Fig. 7*B*). This may be an aggregated form of the receptor.

Comparison of the chromatographic mobility of the cross-linked receptor-ligand complex with that of the unbound receptor suggests that the occupancy of the binding site by its ligand may promote the reversible association of the receptor with additional proteins, possibly other TNF-α receptors, or other membrane or intracellular proteins involved in signal transduction.

Although the method reported here provides only a small amount of purified receptor, continued improvement of the recovery at various stages should provide enough material as antigen for the production of monoclonal antibodies. These antibodies might be valuable reagents for probing the structure and function of various domains of this receptor, isolation of receptor cDNA by expression cloning, and, ultimately, purification of large amounts of receptor. The method described here should also be useful in studies which seek to compare TNF-α receptors among different cell types as well as those studies aimed at determining the cellular events subsequent to ligand binding which might involve receptor modification.

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Supplementary Material to:

HUMAN TUMOR NECROSIS FACTOR-α RECEPTOR: PARTIAL PURIFICATION BY IMMUNOAFFINITY CHROMATOGRAPHY AND INITIAL CHARACTERIZATION

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Experimental Procedures

Materials – Carrier free Na ^{125}I was purchased from Amersham, Arlington Heights, IL. Penicillin, streptomycin, Iscove's Modified Dulbecco's Medium (IMDM), and FBS, were obtained from GIBCO, Santa Clara CA. Triton X-100, Digitonin, and n-octyl β-D-glucopyranoside, aprotinin, bacitracin, phenyl methyl sulfonyl fluoride, PEG 6000, and thimerosol were obtained from Sigma (St. Louis, MO). (3-[(3-cholamidopropyl)dimethyl-ammonio] 1-propane sulfonate) (CHAPS) and Thesit were supplied by Boehringer Mannheim, Indianapolis, IN. IODO-GEN and the crosslinking reagents EGS, sulfo-EGS, DSS, DSP, and DTSS were obtained from Pierce Chemical Co, Rockford, IL. Molecular weight standards for gel filtration, PD 10 columns, protein A-Sepharose, and CNBr Sepharose were obtained from Pharmacia, Piscataway N.J.

Escherichia coli derived recombinant human tumor necrosis factor-alpha (rTNF-α) purified to homogeneity was kindly supplied by Dr. E. Rinderknecht of Genentech Inc., So. San Francisco (2). The specific activity of this cytokine as determined by lysis of actinomycin D treated mouse fibroblast L-M cells (27) was 5×10^7 U/mg. Rabbit polyclonal anti-TNF-α antibodies were obtained as described previously (28) and the neutralization titer of this antibody was 9×10^6 units of TNF-α/ml of serum. A subclone of the human histocytic lymphoma cell line, U937 (CRL No. 1593), was kindly supplied by Dr. Glenn Rice of Genentech Inc., South San Francisco (manuscript in preparation).

Radiolabeling of TNF-α –TNF-α was labeled with Na^{125}I using the iodogen procedure as described previously (21,29). Briefly, 10 μg of rTNF-α in 20 μl was layered over a freshly prepared film of iodogen (10 μg), and incubated for 10 min at 4°C in the presence of 1 mCi of carrier-free Na^{125}I. Free iodine was removed by gel filtration on a Sephadex G-25 column equilibrated with PBS containing 0.1% gelatin and 0.01% thimerosol. Further characterization of the iodinated TNF-α was carried out as described earlier (21). More than 90% of the iodine in the final product was incorporated into the protein as determined by both trichloroacetic acid precipitation and analytical SDS-PAGE analysis. The specific activity of the labeled TNF-α was approximately 60 μCi/μg.

Cell Culture – The human histocytic lymphoma cell line U937 was grown in IMDM supplemented with FBS (10%), penicillin (100 μg/ml), and streptomycin (100 μg/ml). The cells were first seeded in T 175 flasks filled with 150 ml of cells at a density of about 0.25×10^6/ml and grown at 37°C under an atmosphere of 95% air and 5% CO_2. When the density reached approximately 10^6 cells/ml, 150 ml of cell suspension was then transferred to two liter roller bottles with an equal volume of fresh medium. The volume was doubled every 2 days by addition of fresh medium until the final volume was 1.75 liters. The cells were harvested when the density reached between $0.8–1.0 \times 10^6$ cells/ml.

Assay for TNF-α Receptor on Intact Cells – U937 cells (10^5 cells/0.1 ml/tube) resuspended in binding buffer, (IMDM supplemented with 10% (v/v) FBS), were incubated with a variable concentration of ^{125}I-TNF-α in the presence or absence of 100-fold excess unlabeled TNF-α for 2 h at 4°C. Cells were collected by centrifugation at 2,000 x *g* for 5 min and washed three times with 1 ml of ice cold binding buffer. Cell bound radioactivity was measured using a Micromedic 4/600 γ counter. Nonspecific binding, determined in the presence of unlabeled TNF-α, was 5–10% of the total binding. Specific binding was determined by subtracting nonspecific binding from total binding. Each determination was made in triplicate.

Solubilization of TNF-α Receptor – Approximately 10^8 U937 cells were suspended in 1 ml of solubilization buffer consisting of 50 mM Tris, pH 7.5, supplemented with aprotinin (0.2 mg/ml), PMSF (1 mM), and bacitracin (0.1%) and a given detergent, at 1% (v/v) final concentration. After mixing gently for 30 min at 4°C, the extract was then diluted ten-fold with solubilization buffer (minus detergent) and centrifuged at 200,000 x *g* for 1 h at 4°C. The solubilized receptor was recovered as a clear supernatant.

Assay for Solubilized TNF-α Receptors – Detergent solubilized cell extracts were incubated at 37°C for 30 min with ^{125}I-TNF-α, in the absence or presence of 100-fold excess unlabeled ligand in a total volume of 0.14 ml. The buffer was 50 mM Tris, pH 7.5, and contained 0.65 M NaCl, 0.018% (w/v) bovine gamma globulin, and 0.1% Triton X-100. Subsequently, an equal volume of PEG 6000 (22% in PBS) was added and after an additional incubation for 10 min at 4°C, the receptor ligand complex was pelleted by centrifugation at 1000 x *g* for 30 min. The supernatant was removed by aspiration and the radioactivity in the pellet was counted directly in a Micromedic 4/600 γ counter. Specific binding was calculated by subtracting the nonspecific binding from total binding. Although less than 5% of labeled TNF-α in buffer was precipitated under these conditions, in the presence of cell extract, the non-specific binding for this assay approached 30-40%. Therefore the assay was used primarily to follow activity, rather than to quantitate it precisely.

Covalent Crosslinking of TNF-α to its Receptor – The entire procedure was carried out at 4°C. Intact cells, (10^7/ml) were incubated with TNF-α (10 nM), washed three times with binding buffer to remove unbound ligand, and then washed twice with phosphate buffered saline (PBS) to remove serum proteins prior to crosslinking. The washed cells were resuspended in ice cold PBS, containing 0.1 mM of crosslinking reagent, freshly prepared in DMSO or H_2O. After incubation for 40 min at 4°C with gentle agitation, the unreacted crosslinking reagent was quenched with 20 mM ammonium chloride (final concentration). After another 10 min, the cells were washed with cold PBS and then solubilized with 1% Triton X-100 as described above.

Specific Immunoprecipitation of Crosslinked TNF-α-Receptor – The receptor, covalently crosslinked to the ligand, was immunoprecipitated using a combination of rabbit anti-TNF-α IgG, and protein A Sepharose. First, the clarified cell extract was preincubated at 4°C for 90 min with protein A sepharose equilibrated in PBS containing 0.1% Triton X-100 (equilibration buffer) to remove proteins which might bind nonspecifically to the resin. The unbound fraction was then incubated with either anti-TNF-α antibody or, as a control, anti bGH antibody (rabbit) for 12 h at 4°C followed by binding to a fresh aliquot of protein-A sepharose for 90 min at 4°C. The immune complexes were collected by centrifugation at 1000 x *g* for 5 min, and washed five times with 20 volumes of equilibration buffer. The receptor-ligand complex was recovered from the pellet by extraction with SDS sample buffer, then analyzed by SDS-PAGE.

Preparation of Anti-TNF-α Immunoaffinity Column – Purified IgG was prepared by passing the antiserum over a TNF-α affinity column (approximately 5 mg TNF-α/ml of Affigel 10) and eluting the antibody with 0.1 M glycine-HCl, pH 2.5. The eluate was neutralized by dialysis against PBS pH 7.5. After dialysis, these antibodies were coupled to sepharose. Cyanogen bromide activated Sepharose CL-4B resin (~0.3 gram) was washed first with 1 mM HCl, then with coupling buffer (0.1 M $NaHCO_3$, 0.5M NaCl, pH 8.3), and then transferred immediately to a second tube containing 3 ml of affinity purified rabbit anti TNF-α IgG (~0.3 mg/ml) in coupling buffer. After 2 h incubation at room temperature with constant mixing, the reaction was stopped by adding 0.2 M glycine, pH 8.0. A 5 ml plastic syringe was packed with 1 ml of resin and washed first with several volumes of equilibration buffer and then with the same buffer containing 6 M guanidine hydrochloride (GuHCl). The column was finally rinsed with equilibration buffer before use. This affinity matrix was stable for several months at 4°C and could be recycled 10 times under the conditions described.

Immunoaffinity Purification of the Receptor – Soluble U937 cell extracts containing the crosslinked ligand-receptor complex (10% attached to labeled TNF-α, 90% to unlabeled) were incubated at 4°C for 12 h on a rotary mixer with anti TNF-α-IgG Sepharose. Thereafter, the resin was repacked into a column and washed first with 500 volumes of equilibration buffer containing 1 mM PMSF, then 100 volumes of 0.5 M NaCl in the same buffer, 50 volumes of 30 mM n-octyl glucoside in PBS, 50 volumes of equilibration buffer, and finally 5 volumes of equilibration buffer containing 1 M GuHCl. The receptor-ligand complex was then eluted with 6 M GuHCl in equilibration buffer containing 1 mM PMSF. The eluted fractions (approximately 10 ml total) were immediately dialyzed against 4 liters of 25 mM Tris, containing 0.1% Triton and 1 mM PMSF, at 4°C for 12 h, then concentrated by precipitation with 5 volumes of ice cold acetone. The precipitates were resuspended with SDS-PAGE sample buffer prior to preparative SDS-PAGE.

Preparative Sodium Dodecyl Polyacrylamide Gel Electrophoresis – Samples containing ^{125}I-TNF-α labeled receptors were electrophoresed in the presence of 0.1% SDS on 5-15% acrylamide gradient gels using the method of Laemmli (30) at a constant current of 25 mA. Unless otherwise stated, all samples to be electrophoresed were first reduced with 50 mM dithiothreitol for 2 min at 60°C. Prestained molecular weight standards were used to identify the approximate region of the gel where labeled receptor would be located. After electrophoresis the gel was immediately frozen on dry ice, then 1 mm sections were sliced and counted for ^{125}I-TNF-α. The fractions containing the crosslinked TNF-α-receptor complex were eluted by crushing the slices in the presence of 200 μl of 0.01% SDS, followed by overnight incubation at 4°C. The solubilized receptor was recovered in greater than 95% yield from the supernatant of the gel homogenate by passing the solution through a porous plastic filter.

Analytical Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis – After electrophoresis, gels to be stained were immediately fixed in a solution of 40% methanol and 10% acetic acid. Protein was visualized by staining with silver or Coomassie blue. The gels were dried between cellophane membranes before autoradiography. Radioactively labeled proteins were visualized by exposing the dried gel to Kodak X-Ray film with Cronex intensifying screens at -70°C.

Gel Filtration – Detergent solubilized receptor and solubilized ligand receptor complex were also analyzed on a prepacked Superose 6 column using a Pharmacia FPLC system. The column was equilibrated at a flow rate of 0.3 ml/minute in PBS containing Triton X-100 (0.1%) and aprotinin (0.2 mg/ml), PMSF (0.1 mM), and bacitracin (0.1%). The molecular weight standards used to calibrate the column were thyroglobulin (660,000), ferritin (440,000), catalase (236,000), and bovine serum albumin (66,000). Two hundred microliters of a given extract were applied to the column. The binding activity of each fraction was determined using the soluble binding assay described above. For the labeled, crosslinked receptor, the fractions were counted directly in a Micromedic 4/600 γ counter. Selected fractions were then analyzed by SDS-PAGE and autoradiography.

Protein Determination – The protein content of various fractions was estimated using a modification of the method of Bradford (31). Briefly, 25 μl of the sample to be tested was diluted with 775 μl of PBS and then mixed with 200 μl of protein assay dye reagent concentrate as supplied by Bio Rad. The concentration of protein was measured by monitoring the absorbance at 595 nm and comparing these values to a standard calibration curve using BSA as a standard.